JOURNAL OF BONE AND MINERAL RESEARCH
Volume 18, Number 8, 2003

Dentin Matrix Protein 1 Is Expressed in Human Lung Cancer

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ABSTRACT

We have previously shown that breast and prostate cancers express bone matrix proteins. DMP1 expression was evaluated in 59 human lung cancer samples at the protein and mRNA levels. It was detectable in 80% of the cases, suggesting a potential role for DMP1 in tumor progression and bone metastasis.

Introduction: Previously, we and others have shown that bone extracellular matrix proteins such as bone sialoprotein (BSP) and osteopontin (OPN) are expressed in various types of cancer that are characterized by a high affinity for bone including breast, prostate, and lung adenocarcinoma. Based on biochemical and genetic features, BSP, OPN, dentin matrix protein 1 (DMP1), and dentin sialophosphoprotein (DSPP) have been recently classified in a unique family named SIBLING (small integrin-binding ligand, N-linked glycoprotein). Therefore, we investigated whether DMP1 could also be detected in osteotropic cancers.
Materials and Methods: We first used a cancer array for evaluating the relative abundance of DMP1 transcript in a broad spectrum of human cancer tissues. This screening showed that DMP1 was strongly detectable in lung tumors compared with normal corresponding tissue. In a second step, we used an immunophosphatase technique and a specific polyclonal antibody directed against DMP1 to examine the expression of DMP1 in 59 human non-small cell lung cancer samples, including 29 squamous carcinoma, 20 adenocarcinoma, and 10 bronchioloalveolar carcinoma. Student's *t*-test was used to determine the statistical significance of immunostaining scores between the lung cancer histological groups studied and between cancer and normal lung tissues.
Results: Our results show that DMP1 is detectable in 90% of the adenocarcinoma and squamous carcinoma analyzed while 8 of 10 bronchioloalveolar specimens were negative. DMP1 immunostaining intensity and extent scores were significantly higher in adenocarcinoma ($p = 0.0004$) and squamous carcinoma ($p < 0.0001$) samples compared with adjacent normal lung tissue. In situ hybridization experiments confirmed that DMP1 mRNA is localized in lung cancer cells.
Conclusion: In this study, we show that a third SIBLING protein is ectopically expressed in lung cancer. The role of DMP1 in lung cancer is largely unknown. Further studies are required to determine the implication of this protein, next to its sisters SIBLING proteins, in tumor progression and bone metastasis development.
J Bone Miner Res 2003;18:1506–1512

Key words: dentin matrix protein 1, bone metastasis, lung cancer

INTRODUCTION

Dentin matrix protein 1 (DMP1) is a noncollagenous extracellular matrix protein that was originally isolated from dentin and has since been found in other calcified tissues such as calvaria and long bone.[1,2] DMP1 is genetically and structurally related to other acidic proteins found entrapped within the mineralized matrices of bones and teeth including bone sialoprotein (BSP), osteopontin (OPN), dentin sialophosphoprotein (DSPP), and the more distantly related basic protein, matrix extracellular phosphoglycoprotein (MEPE). Recently, Fisher et al. has grouped BSP, OPN, DMP1, DSPP, and MEPE in a unique family, the SIBLING proteins family.[3] These proteins are all made by osteo-

The authors have no conflict of interest.

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blasts and odontoblasts and share common properties: (1) they are phosphorylated sialoglycoproteins; (2) the genes coding for these proteins have similar exonic structure and are all clustered on human chromosome 4; and (3) each contains the integrin-binding RGD tripeptide. The acidic SIBLINGs are all thought to bind strongly to hydroxyapatite, the mineral found in bones and teeth. BSP and OPN have both been shown to be entirely flexible in solution, a property often found for proteins that have many binding partners.[3] The function of DMP1 is not known this time.

Lung cancer belongs to the group of malignant lesions that specifically select bone as secondary implantation site. Lung carcinoma are classified into two major histological subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), according to the World Health Organization classification. Each type seems to have different specific biological, clinical, and prognostic features. SCLC metastasizes to systemic organs very rapidly and is associated with poor prognosis despite multimodality therapy. Patients with NSCLC are usually subjected to surgery; however, the 5-year survival rates are low.

We and others have previously shown that two SIBLING members, BSP and OPN, are highly upregulated in various types of cancers that are characterized by their high affinity for bone, such as breast, prostate, and lung adenocarcinoma. In this study, we hypothesized that another member of the SIBLING family, DMP1, may also be expressed in lung cancer. We first looked at DMP1 expression using a cDNA cancer array. We then performed immunohistochemistry experiments on a series of primary NSCLC and normal corresponding lung tissue. Finally, we used an in situ hybridization (ISH) technique to assess endogenous production of DMP1 mRNA in lung cancer cells.

MATERIALS AND METHODS

Cancer profiling array

cDNA array hybridization was performed with the use of a commercially available membrane (Cancer Profiling Array; Clontech laboratories, Palo Alto, CA, USA). All information regarding the preparation of the cDNAs and the membrane are fully described in the user manual. The coding portion of human DMP1 exon 5 was amplified from human genomic DNA by specific oligonucleotides and subcloned. The sequence-verified insert was purified and labeled with [α-^{32}P]dCTP by random priming. After removal of unincorporated isotope, the labeled probe was denatured and hybridized to the blot according to the manufacturer's instructions. After washing under high stringency conditions, the blot was visualized using a PhosphorImager (ABI). Duplicates blots were probed with cDNA inserts encoding human BSP clone B6–5g[4] and human OPN cDNA clone OP-10.[5] We analyzed a set of 42 cDNAs consisting of 21 lung tumor samples including 11 squamous, 1 adenosquamous, 4 adenocarcinomas, 3 bronchioloalveolar, 2 malignant carcinoid tumors, and 21 samples from the corresponding normal tissues.

Production of antiserum against human DMP1 (LF-148)

Two synthetic peptides (CEHPSRKIFRKSRISE and CLKNIEIESRKLTVDAYH were conjugated through the Cys group to activated keyhole limpet hemocyanin (Pierce) and injected into a New Zealand white rabbit under an approved animal care protocol in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facility. Both peptides seemed to result in active antibody production as determined by binding activity of the antiserum to specific small fragments of human DMP1 protein made in *E. coli*. LF-148 also cross-reacted with authentic recombinant bovine DMP1 made in human marrow fibroblasts.[6]

Tissue specimens

A total of 59 human lung cancer tissues were obtained from the Department of Pathology of the University of Pisa, Pisa, Italy. Specimens were fixed in formalin, embedded in paraffin, and cut into 5-μm sections. The human lung tissues examined included 20 adenocarcinoma, 29 squamous carcinoma, and 10 bronchioloalveolar cancers. Adjacent normal lung tissue was examined when present. Four additional frozen tissue samples from lymph nodes invaded by lung carcinoma cells, obtained from the Department of Pathology at the University Hospital of Liège (J Boniver), were used for DMP1 mRNA ISHs.

Immunohistochemistry

Immunophosphatase staining was performed with the use of the ABC Vectastain Kit (Vector Laboratories, Inc., Burlingame, CA, USA) according to the supplier's direction and LF148 anti-DMP1 rabbit polyclonal antibody. Briefly, sections were deparaffinized in xylene and rehydrated through a graded ethanol series. For antigen retrieval, slides were heated in a water-bath at 95°C for 40 minutes in 10 mM citrate buffer (pH 6). After the endogenous phosphatase activity was blocked with Levamisole 0.4 mg/ml (Sigma) in Tris-buffered saline (pH 7.4) for 5 minutes, the slides were incubated with normal goat serum (1:20) for 30 minutes to block the nonspecific immunoglobulin staining serum-binding sites. Anti-DMP1 LF148 at a dilution of 1:1000 was applied and incubated overnight at 4°C. The slides were then incubated with biotinylated goat anti-rabbit antibody followed by the alkaline phosphatase-conjugated streptavidin complex. All reagents were diluted in Tris-buffered saline and incubated for 30 minutes at room temperature. Washes were performed after each incubation step. Phosphatase activity was revealed by the New Fuchsin substrate-chromogen system (DAKO), and the color reaction was allowed to develop for 20 minutes. Finally, the slides were washed in tap water, counterstained with hematoxylin, and mounted with coverslips. Negative controls for immunostaining were obtained by substituting the first antibody with normal goat serum, and bronchial cartilage was used as an internal positive control.

Evaluation of immunohistochemical staining

The immunohistochemically stained sections were reviewed by two independent observers. Tumor samples were subclassified with regard to immunostaining intensity and extent scores according to similar semiquantitative scales that ranged from 0 to 3. For staining intensity, 0 represented negative samples; 1+, a weak staining; 2+, an intermediate or moderate staining; and 3+, a strong staining. When the tumor showed some heterogeneity in the intensity of the staining, the scoring of the intensity was assessed according to the staining of the most positive tumor cells, when their estimated percentage represented at least 30% of the total positive tumor cell area.[7] For staining extent, 0 represented samples in which DMP1 expression was undetectable, 1+ denoted samples in which up to 33% of the tumors exhibited a detectable level of anti-DMP1 immunoreactivity, 2+ signified those with detectable staining in more than one-third to two-thirds of the tumor cells, and 3+ represented those in which more than two-thirds of the tumor cells expressed a detectable level of DMP1 expression. To provide a global score for each case, the results obtained with the two scales were multiplied, yielding a single scale with steps of 0, 1+, 2+, 3+, 4+, and 6+, as described previously.[7]

ISH

The template used was the full-length human DMP1 cDNA cloned in the Stratagene pBluescript SK vector. After linearizing the plasmid with the appropriate restriction enzymes (*Bam*H1 and *Eco*RI for antisense and sense probes, respectively), we obtained a digoxigenin (DIG)-labeled single-stranded antisense RNA probe by using the T3 RNA polymerase and a DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany). Similarly, a sense digoxigenin-labeled RNA probe was prepared for negative control experiments by the use of T7 RNA polymerase and the same labeling kit. Frozen sections were fixed for 15 minutes in 4% paraformaldehyde in PBS. After washing in PBS, the slides were dehydrated through a series of incubations in 30%, 50%, and 70% ethanol for 5 minutes each. After a 15-minute wash in PBS, tissues were acetylated for 10 minutes in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) and subsequently rinsed in 0.1 M glycine, 0.1 M Tris (pH 7.0) for 10 minutes. The sections were dehydrated in increasing ethanol concentrations and air dried. Approximately 30 μl of hybridization buffer containing 30 μg/ml of DIG-labeled RNA probes (sense or antisense) were applied to each section. The hybridization reaction was carried out at 55°C overnight in the hybridization buffer (50% deionized formamide, 5× SSC, 10% dextran sulfate, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml ssDNA). After hybridization, the sections were washed two times (6 minutes each) in 2× SSC/0.1% SDS and then two times (15 minutes each) in 0.1× SSC/0.1% SDS at 55°C. The tissues were treated with 40 μg/ml RNase A at 37°C for 30 minutes. After washing with 2× SSC for 2 minutes, the sections were incubated with a sheep alkaline phosphatase-conjugated anti-digoxigenin antibody for 30 minutes. The alkaline phosphatase reaction was developed by addition of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Finally, sections were counterstained with methyl green and mounted under coverslips for microscopy examination.

Statistical analysis

Student's *t*-test was used to determine the statistical significance of immunostaining scores between the lung cancer histological groups studied and between cancer and normal lung tissues. A p value <0.05 was considered statistically significant. The analyses were carried out using the StatView software version 5.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

We used a commercially available cancer cDNA array as a tool to perform a preliminary screening of the relative levels of DMP1 gene expression in human tumors. The lung portion of this array comprised a set of 21 lung tumor cDNAs and the corresponding normal tissue from the same individuals that allowed us to compare DMP1 expression between the tumor and corresponding normal lung. Although, DMP1 expression was also found in other tumor types, we decided to focus our study on lung tumors because the differential expression in malignant lung tissue versus the corresponding normal tissue was the most evident in this tissue. Indeed, the visual evaluation of hybridization signals revealed that the majority of the lung tumors were positive for DMP1 expression, whereas normal corresponding tissue was negative (Fig. 1). The level of expression of DMP1 was found to be quite variable within the different lung cancer samples. We also hybridized duplicate blots using BSP and OPN probes. As expected, they both revealed more positive signals among tumoral tissue than normal tissue cDNAs (Fig. 1). Although tumor specimen expressing high level of DMP1 also generally highly express BSP and OPN, there was not always a strict parallelism in the level of expression of the three SIBLINGs analyzed for the same sample. We noticed that 1 of the 21N lung samples appeared positive for DMP1 (Fig. 1, sample 4), whereas its matching cancer tissue was negative. The observation that this "normal" sample was also positive in both OPN and BSP arrays and that the corresponding "tumor" sample was always negative made us suspect that an inversion may have occurred during the spotting of the array or earlier, when the tissue samples were collected. None of the negative controls present on the array hybridized to DMP1, OPN, or BSP specific probes.

The array findings prompted us to look at DMP1 expression in 59 NSCLCs, including 20 adenocarcinoma, 29 squamous carcinoma, and 10 bronchioloalveolar tumors, using an immunophosphatase technique and a polyclonal anti-DMP1 antibody. Normal lung tissue was found to be consistently negative, as shown in Fig. 2A, or exhibited a very low level of DMP1 expression. Bronchial cartilage was always found to be positive and was thus used as an internal positive control when available on the tissue sections (Fig. 2B). The intensity and extent of the immunostaining for the different types of lung lesions analyzed are summarized in Table 1. Of the 59 lung cancer lesions studied, 47 (79.7%) expressed detectable levels of DMP1 (1+, 2+, or 3+). The

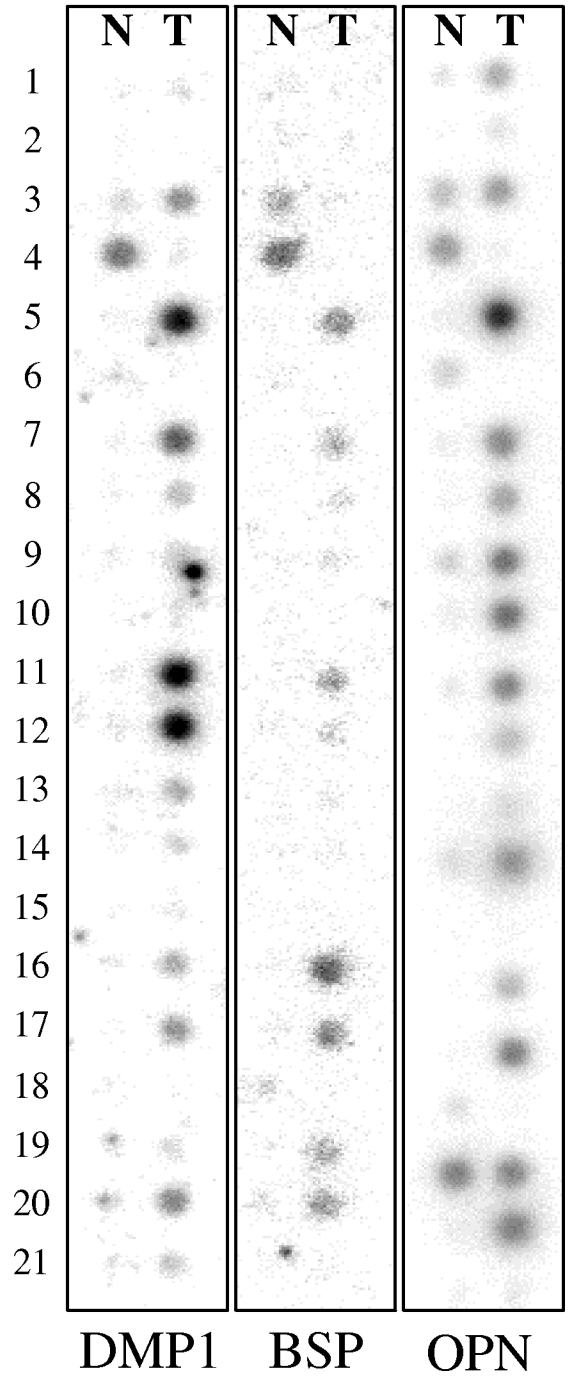

FIG. 1. Phosphorimager images of the lung portion of a Cancer Profiling Array (Clontech) comprising 21 lung tumor samples (T) and 21 samples from the corresponding normal tissues (N) hybridized with DMP1, BSP, and OPN probes, as described in the Materials and Methods section. Visual inspection of the arrays reveals that the majority of lung cancer samples show a higher DMP1, BSP, and OPN than corresponding normal lung samples.

staining was mainly localized in the cytoplasm of the tumor cells as shown in two representative squamous carcinoma lesions in Figs. 1C and 1D (2+ and 1+ immunostaining intensity score, respectively). DMP1 immunoreactivity was remarkable in the tumor cells of adenocarcinoma and squamous carcinoma lesions with 90% of positive tumors in each category, whereas the majority of bronchioloalveolar cancers examined (80%) did not express DMP1 protein. Comparative statistical analyses of DMP1 expression scores among the normal and neoplastic tissues are presented in Table 2. DMP1 expression was significantly higher in adenocarcinoma and squamous carcinoma compared with normal tissue (Student's t-test, $p = 0.0004$ and $p < 0.0001$, respectively). There was no statistically significant difference between bronchioloalveolar cancer lesions and normal lung tissue. Both adenocarcinoma and squamous carcinoma expressed significantly higher levels of DMP1 than bronchioloalveolar cancer tissues (Student's t-test, $p < 0.0001$ and $p = 0.0011$, respectively).

To confirm that DMP1 is detectable at the mRNA level in lung cancer cells, ISH was performed on frozen lung cancer lymph node metastases. DMP1 transcripts were specifically detected in the cytoplasm of infiltrating lung cancer cells (Fig. 2E). The negative control, to which the sense riboprobe was applied, showed no specific reactivity (Fig. 2F).

DISCUSSION

Cancer commonly metastasizes to bone with up to 80% of breast, prostate, and lung cancer patients developing bone metastases.[8] For the past few years, our group has been interested in the study of bone matrix proteins' expression in so-called osteotropic cancers. NSCLC accounts for about 75–80% of lung cancer cases and carries a 5-year survival rate of about 10–15% for all stages. Surgical resection is the treatment of choice for patients with stage I or II cancer, whereas patients with later stages of disease are treated with combinations of surgery, chemotherapy, and radiation therapy, all of which have significant side effects.[9] Bone is one of the devastating metastatic sites in lung cancer, and nearly all patients with bone metastases die within 1 year.[10]

We and others have previously reported that BSP[11] and OPN[12,13] are frequently expressed in human lung cancer. High OPN expression in lung tumors was found to be an indicator of poor prognosis.[12] In agreement with these observations, serum levels of BSP and OPN were substantially elevated in lung cancer patients compared with healthy subjects.[14] We have shown in this study that another member of the SIBLING family, DMP1, is also expressed in lung cancer cells at the protein and mRNA level. Our preliminary observation was based on the analysis of a commercially available cancer tissue array hybridized with a DMP1 cDNA probe. Among the large variety of cancer tissue types examined, lung cancer seemed to be the one that more consistently overexpressed DMP1. Different levels of expression of this SIBLING were observed between the lung cancer samples. Because the array was performed using total tissue extract mRNA, these differences are more likely related to cell tissue heterogeneity within the different tumors rather than to different level of cell expression. In addition, because the array manufacturer did not provide specific information regarding the tumor samples, no correlation can be drawn from this experiment. When the same array was hybridized for the detection of BSP and OPN

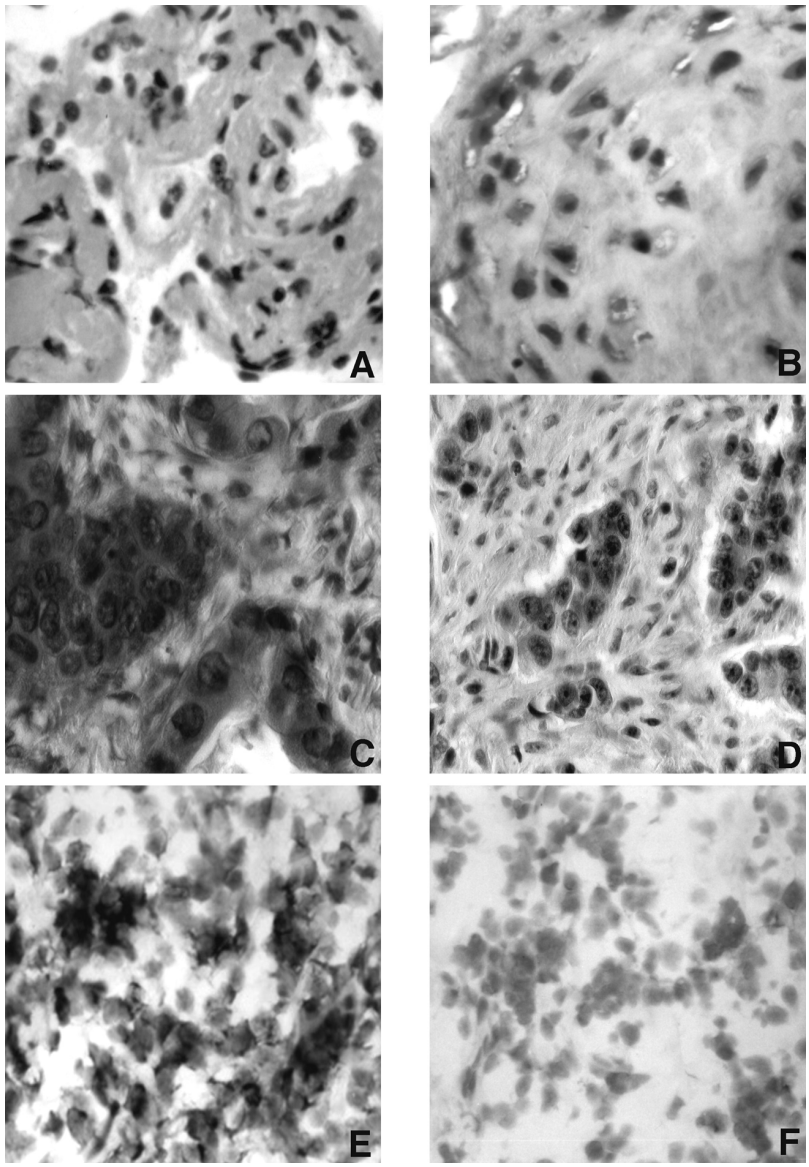

FIG. 2. Detection of DMP1 in human lung cancer by immunostaining and in situ hybridization. Paraffin-embedded tissue sections were immunostained with LF148 polyclonal antibody and counterstained with hematoxylin as described in the Materials and Methods section. (A) DMP1 negative normal lung tissue. (B) Positive bronchial cartilage considered as an internal positive control. (C and D) Representative lung squamous carcinoma exhibiting (2+) and (1+) DMP1 expression, respectively. (E) In situ hybridization on a representative lymph node sample invaded by lung carcinoma cells using antisense probe, as described in the Materials and Methods section, shows DMP1 mRNA transcripts in lung cancer cells. (F) No specific hybridization signal could be detected in a parallel section using sense probe.

mRNA levels, we found in some tumor samples that the expression of these two SIBLINGs did not parallel DMP1 expression. Such discrepancies could be related to different regulatory mechanisms commanding the expression of SIBLING proteins in different lung cancers. Consistently, this study and previous immunohistochemical studies indicate that the majority of lung tumors express BSP, OPN, and DMP1, whereas normal lung tissues do not. Both adenocarcinoma and squamous cancer cells expressed significantly higher amounts of DMP1 than the matching normal lung tissue. In contrast, bronchioloalveolar carcinoma showed a low DMP1 immunostaining intensity that was not significantly different from that observed in normal lung tissue. Bronchioloalveolar carcinoma is a subtype of adenocarcinoma of the lung and carries a better prognosis.[15] In our previous study, we found that BSP was also not detectable in bronchioloalveolar carcinoma. However, our series was comprised of only two samples of this lung carcinoma subtype.[11] Differential expression of OPN has been recently reported between NSCLC and SCC, but no bronchioloalveolar carcinoma samples have been analyzed.[13] It will be interesting to evaluate in further clinical correlative studies whether elevated levels of DMP1 in lung tumors may be associated with the histological type and/or with patients prognosis and outcome.

Experimental evidence suggests that both BSP and OPN may play multiple roles in promoting tumor progression and bone metastasis development.[16–18] We have proposed that cancer cells may use these RGD-containing proteins to adhere to extracellular bone matrix through their cell surface $\alpha v\beta 3$ integrin receptors.[19] A recent study has validated the RGD sequence of DMP1 as a functional motif mediating cell adhesion of several cell types. However, the authors did not show that integrin-type cell surface receptors were involved in this response.[20] The potential role of $\alpha v\beta 3$ receptors in bone metastasis is supported by the

Table 1. Immunohistochemical Analysis of 59 Lung Specimens With a Polyclonal Antibody Directed Against Dentin Matrix Protein 1 (LF148)

Lung lesions	n	*DMP-1 immunostaining intensity*			
		0	*1+*	*2+*	*3+*
Adenocarcinoma	20	1 (5)	14 (70)	4 (20)	1 (5)
Squamous cancers	29	3 (10)	18 (62)	8 (28)	—
Bronchioloalveolar cancers	10	8 (80)	2 (20)	—	—
Total	59	12 (20)	34 (58)	12 (20)	1 (2)
		DMP-1 immunostaining extent			
Adenocarcinoma	20	1 (5)	6 (30)	3 (15)	10 (50)
Squamous cancers	29	3 (10)	10 (35)	4 (14)	12 (41)
Bronchioloalveolar cancers	10	8 (80)	1 (10)	0	1 (10)
Total	59	12 (20)	17 (29)	7 (12)	23 (39)

Numbers in parentheses indicate the percent of specimens in each category.

Table 2. Comparative Statistical Analysis of Dentin Matrix Protein 1 Expression Among Normal and Neoplastic Lesions of the Lung Using Student *t*-Test

Analyzed I × E values	p
Adenocarcinoma vs. bronchioloalveolar cancer	<0.0001
Adenocarcinoma vs. squamous carcinoma	0.4303
Adenocarcinoma vs. normal	0.0004
Squamous carcinoma vs. bronchioloalveolar cancer	0.0011
Squamous carcinoma vs. normal	<0.0001
Bronchioloalveolar cancer vs. normal	0.2824

I × E value represents the multiplied score of intensity and extent values as described in the Materials and Methods section.

$p < 0.05$ was considered as statistically significant.

demonstration of an enhanced expression of this integrin in tumor cells at metastatic sites in patients with bone secondaries.[21] In general, the roles of integrin $\alpha v\beta 3$ include invasion of tumor cells, osteoclast activation in lytic bone metastases, and neovascularization. As potential RGD-ligands of this integrin, we can speculate that the SIBLINGs may contribute substantially to all these functions. Therefore, it is possible that production of bone matrix proteins by cancer cells could enhance development of bone metastases by mechanisms other than facilitating the binding of tumor cells to bone matrix. Recent experimental works show that BSP, OPN, and DMP1 are able to bridge the complement Factor H to cell surface receptors, thereby protecting them from lysis by the alternate complement pathway.[6,14] Through this mechanism, it has been shown that the expression of BSP, OPN, and DMP1 in tumor cells may provide a selective advantage for survival and evading host surveillance.

The exact role of tumor-related bone matrix proteins in the pathogenesis of bone metastases is still unknown, but they may enhance tumor growth, tumor-associated angiogenesis, activation of matrix metalloproteinases (MMPs), protection from immune surveillance, cell-to-bone adhesion, and/or migration into the bone microenvironment. One of the most lethal aspects of cancer arises from its ability to invade and metastasize. Determining the factors that promote cancer cell invasion and metastasis is therefore critically important in treating this disease. Considering all the data describing the involvement of BSP and OPN in different steps leading to tumor invasion and metastasis, we speculate that forthcoming studies on DMP1 will unveil the potential implication of this protein in cancer. Altogether, these studies will help identify potential common mechanism(s) that lead(s) a cancer cell to acquire an osteotropic phenotype through the expression of SIBLING proteins.

ACKNOWLEDGMENTS

The authors thank J Boniver (Head of the Pathology Department, University of Liege) for providing lymph nodes material and P Heneaux and S Thonard for excellent technical assistance. A Bellahcène, L de Leval, and D Waltregny are "Research Associates," and C Detry is a "Télévie Research Fellow" of the Belgian Fund for Scientific Research.

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Received in original form July 7, 2002; in revised form January 9, 2003; accepted February 5, 2003.